Bioavailability of Organic Nitrogen from Treated Wastewater

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STAC Publication 07-001

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Publication Date:
February 2007

Publication Number:
07-001

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STAC Administrative Support Provided by:
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A. Summary

The United States Environmental Protection Agency (EPA) requested guidance from the Scientific and Technical Advisory Committee (STAC) of the Chesapeake Bay Program regarding the bioavailability of organic nitrogen (ON) released through wastewater treatment plant effluents (effluent organic nitrogen or EON) and the appropriateness of a proposed assay for assessing its bioavailability. According to Virginia law, dischargers can argue cases before a nutrient control board to increase their discharge allowances or caps based on their assessment of EON bioavailability. A facility in Virginia employed a bioassay similar to a biochemical oxygen demand (BOD) assay in an attempt to demonstrate that a large fraction of their EON was biologically unavailable. In the short term, EPA requested guidance on: 1) whether EON is bioavailable in the proximate and ultimate receiving waters, and 2) whether the assay employed by the Virginia facility is appropriate for assessing EON bioavailability. In the longer term, the EPA has sought guidance on developing appropriate assays of EON bioavailability.

To address this request, STAC formed an *ad hoc* committee of experts, including wastewater engineers, biogeochemists, and estuarine ecologists, who have prepared this document. This team has found that:

- Many components of dissolved organic N (DON) are indeed bioavailable to microorganisms (including phytoplankton, cyanobacteria, and bacteria) living in estuaries either directly or after physical, chemical, and biologically-mediated reactions in the receiving waters and during transport along an estuarine gradient.
- The assay employed by the discharger for assessing bioavailability of EON is not an appropriate test of bioavailability in the proximate and ultimate receiving waters. There is likely to be a refractory component of EON, but the proposed bioassay would not accurately determine the fraction of bioavailable EON. We lack the scientific information to make such an assessment at this time using any "standard" bioassay technique. We also lack some of the necessary information on both the composition of EON, variations in EON bioavailability from different upstream treatment process configurations, and the transformations it may undergo as it moves from freshwater, through the estuarine gradient, and into the ocean.
- A number of important physical, chemical, and biological factors must be considered in the development of appropriate bioassays.

This document summarizes the scientific background information that led to these conclusions, reviews the reasons the proposed bioassay is considered inappropriate, outlines the factors that need to be considered in developing appropriate bioassays, and identifies gaps in our knowledge currently impeding the development of appropriate bioassays.

B. Background

Nitrogen (N) in wastewater treatment plant effluent includes inorganic and organic forms. Coupled nitrification/denitrification systems (the most common systems) can remove more than 95% of dissolved inorganic N (DIN) (Grady et al. 1999); therefore, a substantial fraction of the residual N in wastewater effluents from biological N removal facilities may be organic. The conventional coupled nitrification/denitrification systems have the potential to remove total N down to 8 mg L^{-1} and, in selected cases, down to 5 mg L^{-1} routinely (Grady et al. 1999). Recent studies have shown that the organic N (ON) remaining in the effluents of biological nutrient removal (BNR) processes, associated with the main wastewater stream, is typically about 1 mg

L^{-1} as N (Murthy et al. 2006). Newer and more expensive technologies must be employed to achieve total N levels of 3 to 4 mg L^{-1} or lower (e.g., Fleishcher et al. 2005) - the anticipated regulatory level for wastewater treatment plants in the Chesapeake Bay watershed (draft Virginia regulations).

Currently there is a growing interest in using novel N removal technologies, such as nitritation/denitrification or nitritation/anaerobic ammonia oxidation (anammox), to remove reduced organic and inorganic N from reject water streams generated by biosolid stabilization processes and recycled to the main wastewater stream in treatment plants, because this recycling can increase the N mass load on the mainstream process by 25 to 40% (Grady et al. in press). This is believed to be a cost-effective way to achieve a total effluent N concentration of 3 mg L^{-1}; however, the impact of these novel N removal strategies on the EON fraction is unknown because they are not yet widely implemented. Nevertheless, implementation by two major wastewater treatment contributors in the Chesapeake Bay region is possible. It is reasonable to expect that different treatment technologies will discharge different amounts and types of residual EON that will be released to the Chesapeake Bay watershed.

Reducing total N in effluents to under 3 mg L^{-1} is expensive. The regulated community is unsure whether reduction beyond that currently realized using conventional methods provides substantial environmental benefits relative to the costs incurred. A significant portion could be ON inert to the biological processing currently employed and that the regulated community contends is biologically refractory in the environment based on the bioassay technique employed. Nevertheless, the bioavailability of ON in wastewater treatment plant effluents has not been widely assessed, nor has its impact on the Chesapeake Bay ecosystem been adequately evaluated, for a number of reasons.

The origin and composition of EON is largely unknown, but is thought to be comprised largely of amides (Dignac et al. 2000a and b). It is also possible that a significant EON fraction is derived from metabolic products generated by the microbes in the wastewater treatment process itself (Parkin and McCarty 1987a and b). It is likely that the various types of wastewater treatment processes will impact EON differently; therefore, it may be necessary to identify the composition of EON generated by each type of process. Finally, ON availability has not been widely examined in freshwater systems because phosphorus (and not N) is more commonly thought to be the limiting nutrient in "fresh" (i.e. non-saline) receiving waters. In contrast, N is generally limiting in marine and estuarine systems. Because DON can be a large fraction of the total N pool, its availability to microbes has been more widely assessed in estuarine and marine waters (see recent reviews on bioavailability of DON by Antia et al. 1991, Bronk 2002, Berman and Bronk 2003, Bronk and Flynn 2006, Bronk et al. 2006), although no study has focused specifically on EON. In contrast, our knowledge of DON bioavailability in freshwaters, including rivers and wastewaters, is still in its infancy (deBruyn and Rasmussen 2002, Pellerin et al. 2006). The lability of natural dissolved organic matter (DOM) varies across aquatic ecosystem types such that it appears to be more labile in lakes and marine systems and least labile in river systems (del Giorgio and Davis 2003).

Due to the huge economic impact of reducing total effluent N to under 3 mg L^{-1} by point-source dischargers, there is a broad interest in a robust method for differentiating bioavailable from recalcitrant EON. This method must be applicable not only to the proximate receiving waters (that may be freshwater) but also to estuarine systems, and sensitive to changing environmental conditions along the length of the estuarine gradient. Regulatory agencies are currently drafting legislation that will allow dischargers to apply appropriate methods to

ascertain the bioavailability of EON in their waste streams, and based on the outcomes of these assays, modify their discharge allowances. Consequently, two major issues must be resolved: 1) quantifying the percentage of EON (derived from waste streams) that is bioavailable along an estuarine gradient (including changes in salinity and ecosystem structure), and 2) establishing a standard method to distinguish between bioavailable and recalcitrant EON that is representative of environmental and ecological conditions in the receiving waters (both proximate and "downstream"). To be scientifically valid, feasible, and protective of the Chesapeake Bay environment, this method must satisfy: 1) marine, estuarine, and freshwater ecologists, 2) Chesapeake Bay Program modelers, and 3) BNR experts.

C. Objectives

The objectives before the STAC team were:

- To assess the actual bioavailability of components of EON to microbes in the environment,
- To assess the suitability of the method used by a Virginia discharger to assess "bioavailability" of EON to microbes, and
- To determine what concentrations of EON result in impairments to receiving streams and their downstream estuaries (this requires developing appropriate methods and defining the physical, chemical, and biological conditions under which the methods must be implemented to be representative of the environment).

The team addressed the first two objectives but not the third because of knowledge gaps identified below.

D. Knowledge Gaps

1. Estuarine environments

Salt influences the behavior, conformation, and reactivity of DOM as it moves through estuaries (Baalousha et al. 2006). Light (photochemistry) can also alter its bioavailability. Biologically recalcitrant DOM can be converted into bioavailable forms via photochemical reactions and subsequently stimulate N-limited microbial food webs (Vähätalo and Järvinen 2007). Additionally, nitrite and ammonium, to highly bioavailable inorganic N compounds, can be released from DOM through photochemical reactions (Kieber et al. 1999 and Koopmans and Bronk 2002). As an added complication, the effects of light and salt on the reactivity of DOM can be interactive (Minor et al. 2006). Finally, chlorinated EON can generate highly toxic compounds and the impact of introducing those products into receiving streams is not well understood (Pehlivanoglu-Mantas and Sedlak 2006).

There are substantial differences in the cycling of nitrogen (N) and phosphorous (P) along the length of an estuary. While freshwater end-members tend toward phosphorous (P) limitation, marine end-members tend toward N limitation (e.g., Doering et al. 1995, Fisher et al. 1999). Consequently there is substantial downstream transport of N relative to P. Because the Chesapeake Bay, other estuarine systems, and the marine environment are more often N-limited (Boynton et al. 1995, Howarth et al. 1996, Kemp et al. 2005), this N is delivered to waters where N can be growth-limiting and where microbial populations (including algae) are adapted to using a broad spectrum of N compounds reside (Paerl et al. 1995, 2004).

Very limited work has been done to assess the bioavailability of EON in freshwater systems (deBruyn and Rasmussen 2002). In marine and estuarine systems, the composition of DOM affects bacterial growth and systems are highly variable (Hopkinson et al. 1998). The variability

of wastewater DOM composition relative to the growth requirements of "assay" microbes is not well understood. Past studies have found that anthropogenically-derived ON is more bioavailable than forest-derived ON (Seitzinger et al. 2002, Wiegner et al. 2006). Finally, bacteria are not the only microbes that use ON; estuarine and marine phytoplankton can also use ON as a source of N (Mulholland et al. 2002a and 2003, Berman and Bronk 2003, Lewitus 2006). We are still learning the extent of these capabilities in natural systems.

2. Composition of EON

The composition of EON was recently reviewed by Pehlivanoglu-Mantas and Sedlak (2006). In general, only a small fraction of DON (and DOM) in aquatic systems has been characterized (e.g., Benner 2002, Bronk 2002, Carlson 2002). The characterizable fraction of DON includes: proteins, free and combined amino acids, low molecular weight (LMW) aliphatic amines, and urea. All of these compounds are found in wastewater and all transformed differently during wastewater treatment. Other identifiable N-containing compounds detected in EON include chelating agents, pharmaceuticals, and soluble microbial products (SMPs) produced during biological treatment (Pehlivanoglu-Mantas and Sedlak 2006). According to these authors, only about 10% of the DON in effluents is identifiable. Included in the complex group of unidentifiable compounds are humic substances, which can be a source of N to estuarine algae (See et al. 2006) and can release N during photochemical reactions (Bushaw et al. 1996, Kieber et al. 1999, Vähätalo and Järvinen 2007). Functionally, DON can be divided into the high molecular weight (HMW) and low molecular weight (LMW) fractions. LMW DON < 2 kD accounts for about half of secondary treated wastewater effluent DON (Pehlivanoglu-Mantas and Sedlak 2006). These molecular weight fractions likely vary in their reactivity and bioavailability (Amon and Benner 1996). New analytical methods and instrumentation are needed to identify the composition of EON more completely.

3. Proposed method for assessing "bioavailability" of EON to microbes

There is concern about the suitability of the method proposed by the Virginia facility for assessing "bioavailability" of EON. The method uses a 140-day bioassay conducted at 20°C under dark, aerobic conditions. Dissolved/soluble total Kjeldahl N (TKN) is measured before and after incubation relative to total N in the effluent to assess the "bioavailability" of DON. Another endpoint of the assay is the conversion of ON to DIN in the bottle. The 140-day assay is based on the length of time it takes for effluent from the plant to reach the Chesapeake Bay and Atlantic Ocean. These methods are not representative of the receiving waters for the following reasons: 1) assays are conducted in the dark, 2) assays are done without phytoplankton, 3) length of the assays relative to endpoints measured is inappropriate, and 4) salinity effects are not considered.

E. Issues to be considered in developing appropriate bioassays

The STAC team agrees that there are currently no appropriate bioassays to accurately assess the bioavailability of EON in receiving waters through the range of environmental conditions it travels en route its ultimate destination, the ocean. The team suggests five criteria that should be satisfied in any bioassay developed to assess bioavailability and discharge allowances for EON.

1. Light

Photochemical reactions affect the lability of organic material along estuarine gradients (Bushaw et al. 1996, Minor et al. 2006) and readily convert "recalcitrant" compounds into reactive material. Photochemical reactions can release biologically available N from biologically non-reactive DON (Vähätalo and Zepp 2005) or may indirectly affect bacterial growth efficiency, bacterial nutrient demand, and bacterial biomass and respiration (McCallister et al. 2005). Additionally, photochemical reactions can convert DOM to inorganic nutrients such as nitrite and ammonium (Kieber et al. 1999; Koopmans and Bronk 2002).

2. Algae

Dark bioassays do not allow consideration of the role of algae in DON uptake. Algal uptake of DON and components of the DON pool, such as urea and amino acids, can be significant in aquatic environments (Bronk 2002; Mulholland et al. 2002a, 2003; Berman and Bronk 2003, Bronk et al. 2006). In addition, a variety of other identifiable N-containing organic compounds can be used as N sources by algae (e.g., dipeptides – Mulholland and Lee submitted; cyanate – Palenik et al. 2003). Further, humic-bound N can also be available to algae (See et al. 2006) and bacterial reactions can degrade other ON compounds into those that can be readily used by algae (e.g. Berg and Jørgensen 2006). In addition to direct uptake of specific DON compounds, microbes (including algae) can render HMW DON into LMW and usable DON through a variety of extracellular mechanisms (Palenik and Morel 1990; Pantoja and Lee 1994, 1999; Pantoja et al. 1997; Mulholland et al. 1998, 2002a, 2003; Berg et al. 2002; Stoecker and Gustafson 2003). Bulk DON uptake by microorganisms has been examined using a bioassay approach (Berg et al. 2003; Stepanauskas et al. 1999a, b; Wiegner et al. 2006) as well as by synthesizing ^{15}N-labeled DON (Bronk and Glibert 1993).

3. Duration of bioassays

The 140-day bioassay period may merely achieve steady state rather than elicit a net effect. Material flow between particulate and dissolved pools includes uptake and production of both ON and DIN. Bacteria are fully capable of consuming DIN as well as ON. The net effect of long bioassays is simply to cycle N among dissolved and particulate pools in a closed system where there is tight coupling of N reactions. The only portion of a bioassay that can be compared to *in situ* metabolic rates is the initial stage, when the pool of labile ON may still reflect *in situ* conditions (del Giorgio and Davis 2003). Bacteria can also modify dissolved organic matter, making it resistant to further degradation (Ogawa et al. 2001). Thus, long incubation times under closed-bottle conditions likely reflect the accumulation of bacterial products rather than recalcitrance of the starting material. Not only is "dissolved" organic matter operationally defined (size cut-offs of filters), but its lability is also operationally defined. The apparent lability of DOM in bioassays depends on the length of incubation and the initial bioassay conditions, which include temperature, size, and composition of bacterial inoculum, as well as the abundance of other inorganic or growth-limiting nutrients (del Giorgio and Davis 2003). Enclosed bioassays tend to favor opportunistic microbes rather than growth of a diverse microbial community.

The incubation length does not necessarily equal length in terms of nutrient cycling along a lotic aquatic ecosystem (e.g. Mulholland et al. 2002b, Payn et al. 2005). While streams and rivers act as nutrient vectors, transformations, recycling, and uptake occur along their flowpath, thereby influencing nutrient retention or loss from the ecosystem. Further, aquatic systems may be managed to reduce N loading to downstream receiving waters (Peterson et al. 2001).

4. Salinity

Salinity increases along the length of the estuarine transit of the waste stream. Changes in salinity are known to alter the reactivity and bioavailability of DON and affect photochemical reactions (McCallister et al. 2005, See 2003, Minor et al. 2006). In addition, the microbial community (bacteria and phytoplankton) changes along the estuarine gradient (Crump et al. 2004, Marshall et al. 2005) affecting nutrient processing and the functioning of ecosystems. Salinity also influences the conformation of macromolecules such as humic substances (Baalousha et al. 2006). These conformational changes can influence both the abiotic and biotic reactivity of DOM.

5. *Differentiating EON from organic nitrogen formed during bioassay*

In any viable assay system, ON will be regenerated and its bioavailability may be different from that initially added to the assay bottle. If a small change in the concentration of ON is detected over the course of the test, it is possible that recalcitrant ON generated during the test will mask consumption of the bioavailable ON being targeted by the test (Section 3 above discusses potential N cycling during bioassays).

F. Conclusions

The proposed bioassay is unlikely to provide a good measure of EON bioavailability once it reaches the receiving waters and then moves through the estuarine system. This can be a potentially significant problem in estuarine systems where N is limiting and microbial populations capable of directly or indirectly using ON to fuel their growth reside. Future efforts to design such a bioassay should incorporate the five criteria described above.

G. Research needs:

1. Compositional studies of EON are needed to quantify and define its various component fractions and the potential lability of those fractions. These should include EON derived from different treatment technologies and different size fractions of EON. These studies would help determine whether bioavailable EON can be removed through alternative or additional treatment or using size-exclusion technologies such as membrane technologies.
2. Mass balance all N pools in time-course studies within incubation bottles to ascertain whether assay results simply reflect recycling through various dissolved and particular N pools over the course of the assays, and that endpoints are just steady state equilibrium conditions within assay bottles. This would include information on the composition of the ON pool to determine if EON was truly refractory or was being transformed in assays. It is likely that both long and short-term assays are required to adequately assess the bioavailability of EON.
3. Identify model organisms appropriate for different salinity ranges encountered in proximate and ultimate receiving waters.
4. Quantify the abiotic effects of salinity and light on the composition and bioavailability of EON. To accomplish this EON could be added to water of various salinities and its bioavailability assessed at a range of salinities in both the light and the dark. Studies would ideally include changes in the composition of the EON pool due to salinity and production of DIN or labile DON from EON.
5. Examine the bioavailability of abiotically altered EON to appropriate test organisms.

6. Compare results from model bioassay systems with EON addition bioassays done using natural water samples collected from different salinity regimes.

Using more sophisticated technologies, ^{15}N-labeled EON could be synthesized and traced directly in aquatic systems.

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